Are there interactions between acetylcholine- and ATP-induced responses at the level of a visceral sensory ganglion?

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ARTICLE INFO

ABSTRACT

We investigate possible interactions between acetylcholine (ACh)- and adenosine 5′-triphosphate (ATP)-induced responses of petrosal ganglion, where the perikarya of most sensory neurons of the glossopharyngeal nerve are located. Experiments were performed on petrosal ganglia excised from pentobarbitone-anesthetized cats, desheathed and perfused in vitro. Separate applications of ACh and ATP to the exposed surface of the ganglion induced bursts of antidromic potentials recorded from the carotid (sinus) nerve branch of the glossopharyngeal nerve, which frequencies were dependent on the dose of the applied agonists. The simultaneous application of previously determined ED_{50}s of ACh and ATP provoked responses corresponding closely to the simple addition of the responses elicited by the separate application of each agent. Responses usually subsided within 1 min of stimuli application but were followed by periods of refractoriness to subsequent application of the same agent. After determining the timing for recovering from desensitization to the ED_{50}s of ACh and ATP applied separately, ACh was applied while the preparation had been desensitized to ATP and then ATP was applied during desensitization to ACh, but responses obtained were similar to control responses induced by each agent separately. In summary, ACh- and ATP-induced responses of petrosal ganglion neurons are simply additive, followed by a few minute lasting desensitization, but cross-desensitization was not observed. Thus, ACh and ATP seem to operate through independent receptors, activating separate ionic channels, whose coincident currents do not interfere each other.

1. Introduction

The perikarya of the sensory fibers conveyed by the glossopharyngeal nerve are mainly concentrated in the petrosal (Andersh's) ganglion with few perikarya located in the superior (Ehrenritter's) ganglion. The barosensory and chemosensory fibers distributed to the carotid body and sinus through the carotid (sinus, Hering's) nerve have their cell bodies in the petrosal ganglion. It must be noted that an extensive ultrastructural search for synapses in this ganglion (Stensaas and Fidone, 1977) revealed the absence of synaptic contacts upon ganglion cell somata, their perikaryal processes (see Pannese, 2002) and emerging unmyelinated axons, all of which were covered by satellite cell processes.

Since the classical studies of Heymans (see Heymans, 1955), acetylcholine (ACh), and its entire group of nicotinic agonists, are recognized as potent stimulants of carotid body chemoreceptors. Adenosine 5′-triphosphate (ATP) was also shown to produce strong excitation of these chemoreceptors (Anichkov and Belen'kii, 1963), excitation also observed later in carotid bodies perfused/superfused in vitro (Spergel and Lahiri, 1993). Based on the assumption that ACh and ATP act upon receptors located at chemosensory nerve endings of the carotid nerve, the presence of such receptors was searched on the perikarya of these neurons at the level of the petrosal ganglion. Thus, nicotinic ACh receptor (nAChR) subunits (Shirahata et al., 1998) and P2X receptor subunits (Prasad et al., 2001) have been described in the petrosal ganglia. Otherwise, Alcayaga et al. (1998) reported the existence of ACh-induced responses mediated by nAChRs, and later (2000) the presence of ATP-induced responses (presumably mediated by P2X receptors) in petrosal ganglia, particularly on the perikarya of neurons projecting peripherally through the carotid nerve. It is known that other sensory neurons – like those of the myenteric plexus – are endowed with P2X receptors in both peripheral nerve terminals and cell bodies (Bertrand and Bornstein, 2002).

The hypothesis that ACh and ATP operate as co-transmitters between carotid body glomus cells and chemosensory nerve endings has been formulated (Nurse and Zhang, 1999). Does this co-action consist in simple additive processes operating in parallel? Or there is inter-action between nAChRs and P2X receptors? Several interactive processes between these two kinds of ionotropic receptors have been postulated or demonstrated in other preparations: (a) partnership between plasma membrane nAChR and $P2X_2$ channels, resulting in nonadditive cross-inhibitory responses (Khakh et al., 2005); (b) receptors clusters in which ion flux through one channel allosterically inhibits current flow through the other channel (Barajas-López et al., 1998); (c) inhibition of ACh-induced currents by very low ATP concentrations producing little or no currents by themselves (Nakazawa et al., 1995), suggesting that the nAChR contains an inhibitory ATP binding site. Mutual occlusion of P2X receptors and nAChRs has been documented on acutely dissociated sympathetic ganglion neurons (Searl et al., 1998). Thus, data available on other preparations indicate occlusion between ACh- and ATP-induced responses.

Present experiments were intended to study possible interactions between ACh- and ATP-induced carotid nerve responses when these agents were applied to the petrosal ganglion. First, the possibility of cross-enhancement of ACh- and ATP-induced responses was searched for. Secondly, the possibility of cross-desensitization between both types of receptors was explored.

2. Results

Topical applications of ACh to the surface of the petrosal ganglion elicited bursts of action potentials conducted antidromically through the carotid nerve. The maximal frequency of carotid nerve discharges (f_{CN}) attained in these bursts was dose-dependent, as shown in Fig. 1. For data pooled from

8 experiments, the ED_{50} of ACh was 155 μg (95% CI: 120 to 201 μg) and the estimated maximal response was 104.2% (±2.8% SEM). The responses elicited by the larger doses of ACh assayed subsided within 50 s. The maximal f_{CN} evoked by ATP applications also was dose-dependent, their ED_{50} being 121 μg (95% CI: 34–433 μg) and the maximal response attained was 40.4% (±5.4% SEM). Thus, the reactivity of the preparation to ATP was less than half that to ACh, in spite of presenting a similar sensitivity to both drugs. In every experiment, the maximal response was evoked by ACh. Responses produced by ATP were slightly longer than those evoked by similar doses of ACh (see inset of Fig. 1), but those evoked by the larger doses of ATP lasted less than 90 s.

Fig. 2 summarizes the dose–response curves for ACh and ATP determined in 5 experiments, in which the effects of 6 co-applications of ACh 100 μg and ATP 100 μg also were tested in each experiment. These doses were included within the confidence intervals of their respective ED_{50}s, and the resulting response was compared to the responses to separate applications of ACh 100 μg and ATP 100 μg. The f_{CN} evoked in response to the mixture (355 ± 36 Hz; mean ± SD) was very close to the algebraic sum of the individual responses to the agonists (215 ± 26 Hz for ACh and 150 ± 21 Hz for ATP), suggesting that there are no interactions between the drugs effects.

We also searched for desensitization to given doses of either ACh or ATP repeatedly applied at variable intervals. For this purpose, the closest dose to the respective ED_{50} separately determined in each experiment was used. Fig. 3 shows that the preparation was not responsive to ACh given 1 min after
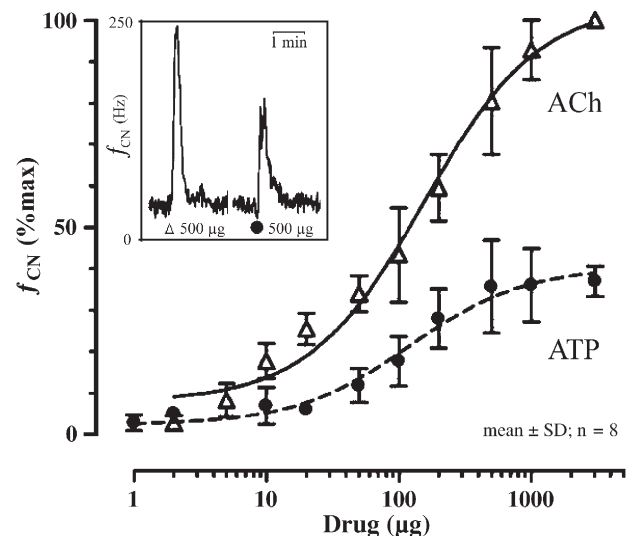

Fig. 1 – Frequency of antidromic discharges recorded from the carotid nerve (f_{CN}) in response to topical application of ACh (triangles and continuous line) and ATP (circles and interrupted line) to the surface of petrosal ganglia superfused in vitro. Responses expressed as percentages of maximal response obtained in each experiment. Means ± SDs from data pooled from 8 experiments. Goodness of fitted curves: r = 0.97 for ACh; 0.73 for ATP. Insert, f_{CN} recordings (0–250 Hz scale) showing responses induced by boluses of ACh and ATP, 500 μg each.
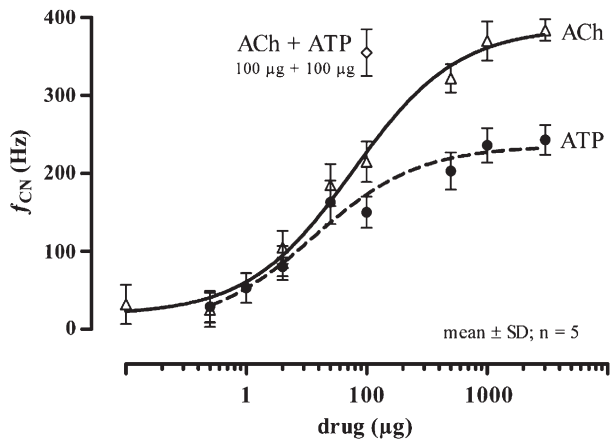
Fig. 2 – Frequency of antidromic discharges recorded from the carotid nerve (f_{CN}) in response to topical application of ACh (triangles and continuous line) and ATP (circles and interrupted line) to the surface of petrosal ganglia superfused in vitro. Combined application of ACh 100 μg and ATP 100 μg (diamond) repeated 6 times in each experiment. Means ± SDs of data pooled from 5 experiments. For ACh: R_{max} = 387 ± 3 Hz (mean ± SEM), ED_{50} = 76 μg (CI 50–117 μg), r = 0.99; for ATP: R_{max} = 235 ± 12 Hz, ED_{50} = 36 μg (13–103 μg), r = 0.98.

its previous administration, and that responses were persistently reduced when given at intervals of 2 and 4 min. Full recovery was only achieved after 15-min intervals (upper abscissa) from previous administration. As shown at the bottom of Fig. 3, f_{CN} values evoked by ACh applications were still significantly below control values in 2 out of 6 experiments when only 8-min intervals were allocated between successive ACh applications, and 15-min intervals were required for full recovery in the 6 preparations tested.

Fig. 4 illustrates the responses elicited by ATP given at the closest doses to the respective ED_{50} determined in each experiment and repeated at different intervals from its first application in each series. Although ATP effects were slightly more prolonged than those of ACh, the response reached a similar f_{CN} than the control one when the dose was given at 5-min intervals from previous applications (as marked in upper abscissa). Similarly, responses tested at 5-min intervals became not significantly different from control responses in all the 6 preparations studied (lower bar at bottom of Fig. 4).

The previous characterization of the time course of ACh and ATP desensitization prompted us to study cross-desensitization between both substances, in the same 6 experiments described before. This is illustrated in Fig. 5. The closest doses to their respective ED_{50}s of ACh, ATP, and ACh again were tested at 1-min intervals, and such sequence was repeated after 30 min. The protocol was repeated 45 min later but this time the order of applications of the ED_{50}s was ATP, ACh and ATP, given at 1-min intervals, the same sequence being repeated after 15-min rest.

The upper part of Fig. 5 shows that, after 1 min of ACh application, an interval during which no response to ACh was expected to occur, the administration of ATP provoked a response of similar magnitude to that expected in control conditions. The confirmation that the preparation was still

desensitized to ACh was revealed by the small increase in f_{CN} elicited when this substance was tested 1 min later. The phenomenon of self-desensitization to ACh without cross-desensitization to ATP was repeatedly observed after the 30-min rest.

The lower part of Fig. 5 shows that during the period when the preparation was desensitized to ATP applications, ACh applications evoked full responses. The increase in f_{CN} provoked by the previously determined ED_{50} of ACh was consistently larger than that induced by the priming ATP application, but it was not different from the control response to ACh. This observation was repeated almost identically when the same sequence of application was retested.

3. Discussion

P2X receptors are widely distributed throughout primary sensory neurons (Burnstock, 2000; Dunn et al., 2001), they are present in the perikarya at the level of sensory ganglia (De Roo et al., 2003), and most rat petrosal ganglion neurons exhibit $P2X_2$ immunoreactivity (Zhang et al., 2000). Otherwise, nAChRs are present in nodose ganglion perikarya (Ashworth-Preece et al., 1998) and four pharmacologically distinct categories of nAChRs have been identified in cultured dorsal root ganglion neurons (Genzen et al., 2001). Similarly, petrosal
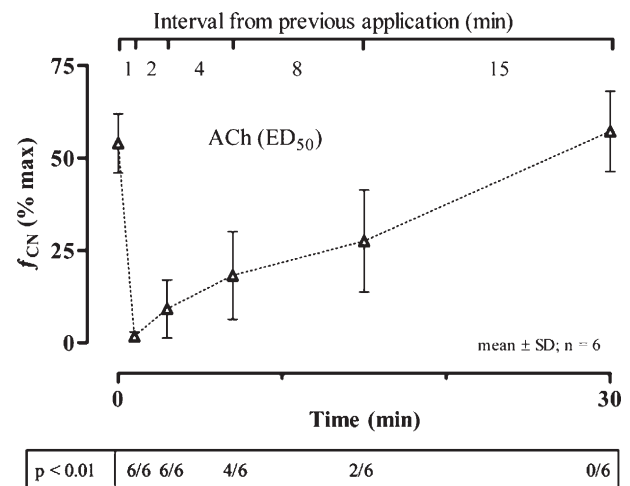

Fig. 3 – Frequency of antidromic discharges recorded from the carotid nerve (f_{CN}) in response to topical application of the same dose of ACh (triangles) to the surface of petrosal ganglia superfused in vitro. Ordinate, f_{CN} expressed in percentage of maximal response evoked in each experiment. The same dose applied in control conditions (time 0 of lower abscissa) was repeated five times, at progressively longer intervals after preceding injection (as indicated in upper abscissa). The dose applied corresponds to the closest to the ED_{50} (from 100 to 500 μg) calculated for each experiment. Means ± SDs. Data obtained from 6 experiments (from the 8 experiments illustrated in Fig. 1). Fractions at the bottom of the figure, number of experiments in which the difference in f_{CN} at that interval with respect to control response was statistically significant (P < 0.01), by two-way ANOVA.
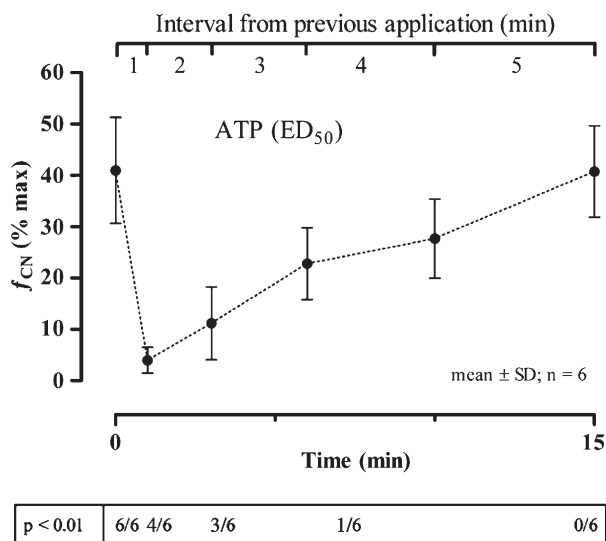
Fig. 4 – Frequency of antidromic discharges recorded from the carotid nerve (f_{CN}) in response to topical application of the same dose of ATP (dots) to the surface of petrosal ganglia superfused in vitro. Ordinate, f_{CN} expressed in percentage of maximal response evoked in each experiment. The same dose applied in control conditions (time 0 of lower abscissa) was repeated five times, at progressively longer intervals after preceding injection (as indicated in upper abscissa). The dose applied corresponds to the closest to the ED_{50} (from 20 to 100 µg) calculated for each experiment. Means ± SDs. Data obtained from 6 experiments (from the 8 experiments illustrated in Fig. 1). Fractions at the bottom of the figure, number of experiments in which the difference in f_{CN} at that interval with respect to control response was statistically significant (P < 0.01), by two-way ANOVA.

ganglion neurons express several nAChR subunits (Shirahata et al., 1998), but electrophysiological recordings and pharmacological data indicate that homomeric α7 nAChRs predominate (Varas et al., 2006). Otherwise, P2X receptor subunits have been described in petrosal ganglia (Prasad et al., 2001) and excitatory responses appear to be mediated by heteromeric $P2X_{2,3}$ receptors (Zhang et al., 2000). Varas et al. (2003), on performing intracellular recordings from somata of petrosal ganglion neurons projecting through the carotid nerve, observed that 24 out of 27 neurons identified as chemosensory units were excited by applications of both ACh and ATP, while two neurons responded only to ACh and just one cell to ATP only. In comparison, none of 10 barosensory neurons was sensitive to ACh and only four of them responded to ATP. This is a strong indication that carotid chemosensory neurons may be characterized by exhibiting both ACh and ATP receptors.

Both nAChRs and P2X receptors are families of ligand-gated ion channels. In petrosal ganglion perikarya, both ACh and ATP exert excitatory actions (Alcayaga et al., 1998, 2000; Zhang et al., 2000; Zhong and Nurse, 1997). Our finding that these excitatory effects are simply additive at this level (Fig. 2) precludes cross-inhibition and suggests that ion currents flow through independent nAChR and P2X channels in the cell membrane. Activation by nicotine and ATP of the same inward currents but through independent ion channels has been described in NGF-

treated PC12 cells (Nakazawa et al., 1991). Pheochromocytoma (PC12) cells are responsive to hypoxia and acidity (Taylor and Peers, 2000) and adrenomedullary cells share many morphological, functional and pharmacological traits with carotid body glomus cells (Fujita et al., 1980; Fearon et al., 2002).

We have confirmed that, while chemosensory (orthodromic) discharges recorded from the carotid nerve upon application of either ACh or ATP to the carotid body can be fully reproduced at intervals of less than 1 min, recordings of antidromic activity from the carotid nerve show that responses elicited by application of ACh and ATP to the petrosal ganglion are followed by intervals of several minutes during which responses cannot be reproduced or are markedly depressed (Alcayaga et al., 1998, 2000). This prolonged desensitization allowed us to perform experiments searching for cross-desensitization between both agents, which should indicate a cross-talk between mechanisms triggered by ACh and ATP receptors. However, the six experiments described in
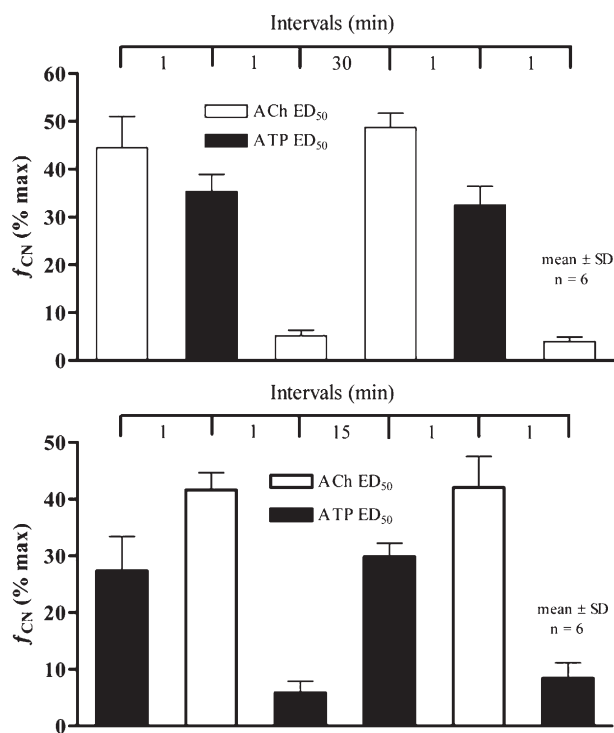

Fig. 5 – Frequency of antidromic discharges recorded from the carotid nerve (f_{CN}) in response to topical applications of ACh (open bars) and ATP (filled bars) to the surface of petrosal ganglia superfused in vitro. Ordinate, f_{CN} expressed in percentage of maximal response evoked in each experiment. Doses applied correspond to the closest to the ED_{50}s previously determined. Means ± SDs. Data obtained from the same 6 experiments illustrated in Figs. 3 and 4. Upper part, ATP applied at 1-min interval after ACh application, period along which expected response to ACh was nearly abolished, followed 1 min later by ACh application producing a small response; sequence repeated after 30-min interval. Lower part, ACh applied 1-min interval after ATP application, period along which expected response to ATP was drastically reduced, followed 1 min later by ATP application producing a small response; sequence repeated after 15-min interval.

Fig. 5 suggest that there is no cross-talk between the effects induced by ACh and ATP when these agents are directly applied to the petrosal ganglion surface.

It may be argued that multiple unit preparations do not allow to affirm that ACh- and ATP-evoked responses were obtained from the same sensory neurons, and that nAChRs and P2X receptors were located on the same perikarya. However, most petrosal ganglion cells projecting to the carotid body through the carotid nerve have been shown to present both types of receptors on the same perikarya, while carotid baroreceptor neurons are devoid of AChRs (Varas et al., 2003). More importantly, the voltage-clamp recordings performed by Nurse and Zhang (2001) on rat petrosal ganglion neurons (co-cultured with carotid body cells) revealed that ACh- and ATP-induced whole cell currents were simply additive. Thus, present observations on the absence of superadditive effects, cross-inhibition and cross-desensitization between ACh- and ATP-induced responses suggest that nAChRs and P2X receptors located on the somata of petrosal ganglion neurons are entirely independent receptors, activating separate ionic channels, and whose coincident currents do not interfere each other.

What is the physiological significance of the presence of ACh and ATP receptors in the membrane of sensory perikarya? Proteins constitutive of membrane receptors and channels are manufactured at the somata of sensory neurons, from where they are axoplasmically transported to sensory nerve endings for their membrane insertion (Koschorke et al., 1994), but they are also inserted in the perikaryal membrane itself. This is the case for nAChR (Polz-Tejera et al., 1980; Ninkovic and Hunt, 1983), while functional P2X receptors are found in the soma and sensory endings, but they are undetectable along the axon (Irnich et al., 2002). In the absence of conventional synapses in the petrosal ganglion (Stensaas and Fidone, 1977), "volume transmission" (Zoli et al., 1998) may operate through substances released from neuronal somata and detected by neighboring cell somata, as proposed for nodose ganglia (Oh and Weinreich, 2002), trigeminal ganglia (Matsuka et al., 2001) and dorsal root ganglia (Amir and Devor, 1996). It must be reminded that centrifugal carotid nerve discharges have been recorded after intracranial section of glossopharyngeal nerve roots (Willshaw and McAllen, 1981), making possible that they were originated from petrosal ganglion perikarya, and therefore corresponding to the carotid nerve antidromic discharges reported in this paper. Although there is no experimental evidence presently available to support the physiological significance of the presence of ACh and ATP receptors in the somatic membrane of sensory ganglia, such presence is certainly useful to advance in their fine characterization. Several histochemical, physiological and pharmacological studies performed on other preparations have shown the close similarities between perikaryal and nerve ending membranes of sensory neurons (Gold et al., 1996; Harper, 1991).

4. Experimental procedures

Preparations were obtained from adult cats, anesthetized with an intraperitoneal injection of sodium pentobarbitone (40 mg/kg) and euthanized at the end of procedures by an overdose of the anesthetic agent, given i.v. On each side, the glossophar-yngeal nerve and its carotid (sinus) nerve branch were dissected. The carotid nerve was cut at its entrance to the carotid body, and the glossopharyngeal nerve was followed up to its entrance to the jugular foramen. The *bulla tympanica* was widely opened, and the ventral wall of the jugular foramen was excised to expose the petrosal and superior (Ehrenritter's) ganglia, which are sometimes fused into a single elongated ganglion. The central process of the glossopharyngeal nerve was cut at approximately 2 mm from the apparent central edge of the ganglion. The ganglion, with its peripheral and central processes, was excised and placed in a Petri capsule with cold Hanks' solution, to remove the capsule of the ganglion and epineurium of its peripheral processes under a stereomicroscope.

The preparation was transferred into a small chamber (0. 2 ml capacity), thermo-regulated at 38.0 ± 0.5 °C, and super-fused at 1.5 ml /min with Hanks' balanced solution supplemented with 5 mM HEPES buffer, pH 7.43, equilibrated with air (20% O_2). The ganglion was pinned to the bottom of the chamber, while the carotid nerve was placed on a pair of recording Pt-Ir electrodes, and lifted to the superior compartment of the chamber, filled with mineral oil. The recording electrodes were connected to an AC preamplifier, and the resulting electroneurogram was displayed on an oscilloscope, digitally encoded and recorded on videotape. The electroneurogram was also fed to spike amplitude discriminator, whose standardized pulses were counted at 1-s intervals to asses the frequencies of antidromic discharges of the carotid nerves (f_{CN}), which were acquired on a computer through an analog-digital interface card, displayed on-line and saved as digital files for later analysis.

Acetylcholine chloride (ACh; Sigma) and adenosine 5'-triphosphate disodium salt (ATP; Sigma) were applied in 10-μl boluses, dissolved in Hanks' solution (Sigma), by means of micropipettes whose tips were placed at about 1-mm distance from the exposed surface of the ganglion. Doses referred to correspond to the salts.

To assess the integrity of the preparation throughout the experiment and the possibility of fiber conduction block during application of drugs, the nerve discharge evoked by electrical stimulation of the petrosal ganglion was recorded from time to time. Brief (50 μs) square electrical pulses were delivered through a pair of Pt-Ir stimulating electrodes placed under the petrosal ganglion and the antidromic compound action potentials recorded from the carotid nerve were analyzed in terms of their latency, amplitude and duration.

Results are expressed as means ± SDs, or as percentages of the maximal response obtained during each experiment, no matter what stimulus caused such response.

Statistical differences for multiple dependent samples were assessed by ANOVA, followed by Bonferroni's test for multiple comparisons. To pool data from different experiments, the dose–response curves were fitted to symmetrical sigmoidal functions (De Lean et al., 1978). Data points were adjusted according to the following logistic expression:

$$\mathbf{R} = \mathbf{R_{max}}/(1 + (\mathbf{ED_{50}}/\mathbf{D})^{\mathbf{S}})$$

where $\mathbf{R}$ = response; $\mathbf{R_{max}}$ = maximal response; $\mathbf{D}$ = arithmetic dose; ED_{50} = median effective dose; $\mathbf{S}$ = slope factor determining

the steepness of each curve. The goodness of each fit was tested by ANOVA, and it was expressed by the correlation coefficient (r), calculated by dividing the variance of the adjusted curve by that of the experimental data.

All analyses were made through GraphPad Prism (GraphPad Inc.) computational program, and differences were considered as statistically significant when $P < 0.05$.

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